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PRINCIPAL INVESTIGATOR: Sigal Gery, Ph.D.

CONTRACTING ORGANIZATION: Cedars-Sinai Medical Center
Los Angeles, CA 90048

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6. AUTHOR(S)
Sigal Gery, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Cedars-Sinai Medical Center
Los Angeles, CA 90048

E-Mail: gerys@schs.org

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TMEFF2 is a novel transmembrane protein-containing two follistatin domains and an epidermal growth factor-like motif-that is mainly expressed in the prostate and brain. TMEFF2 is highly expressed in the AD prostate cancer cell line, LNCaP, and is controlled by androgen. TMEFF2 is also upregulated by estrogen in a dose- and time-dependent manner. Ectopic expression of TMEFF2 can inhibit prostate cancer cell growth. I cloned the 5'-flanking region of the human TMEFF2 gene and using a luciferase reporter assay showed that it contains a functional promoter. The 0.7-kb region upstream to the TMEFF2 transcription start site encompasses the minimal promoter required for TMEFF2 expression. Sequence analysis of the TMEFF2 promoter revealed potential binding sites for several transcription factors including Sp1 and an E-box that could be recognized by c-Myc. An inverse correlation between TMEFF2 and c-Myc expression was found in CWR22 prostate xenografts. Reporter gene and mobility shift assays demonstrated that c-Myc could repress TMEFF2 gene expression through its cognate site. In light of the probable role of TMEFF2 in inhibiting cell growth, its suppression may contribute to the oncogenic properties of c-Myc.

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Annual Report
Year 1
TMEFF2: A novel gene expressed selectively in androgen-responsive prostate cancer
PI: Sigal Gery

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Introduction: Prostate cancer is one of the most frequent causes of cancer related deaths in men. The primary therapy for the disease is hormonal modulation, as the tumor cells usually have a dependency on androgen for growth. However after several years of these therapies, the prostate cancer cells progress of from being androgen-dependent (AD) to androgen-independent (AI) Using subtractive hybridization, I initially searched for genes that were differentially expressed between AD and AI prostate cancers, and identified a gene called TMEFF2 that I found to be selectively expressed in prostate and brain. The purpose of this research is to understand what modulates expression of TMEFF2 and to explore the role of TMEFF2 in the proliferation of prostate cancer cells. These experiments provide the opportunity to define further the molecular mechanisms associated with the progression of prostate cancer that may lead to the development of novel therapeutic targets.

Body:
Task 1 Characterize factors that modulate TMEFF2 expression in prostate cancer cells and evaluate the potential use of TMEFF2 as a serum marker for diagnosis of prostate cancer
a. Test the effect of various hormones and growth factors on TMEFF2 mRNA expression in prostate cancer.

As TMEFF2 is expressed in AD LNCaP cells but not in AI PC3 and DU145 cells, I tested whether TMEFF2 is regulated by androgens. LNCaP cells were grown in media supplemented with charcoal stripped (CS) PBS (to remove steroid hormones) and treated with dihydrotestosterone (DHT). As shown in **Figure 1**, levels of TMEFF2 mRNA were low in cells grown in CS PBS but increased in a time and dose dependent manner upon treatment with DHT.

Genes regulated by androgens can often be induced by ligands of other nuclear hormone receptors. Therefore, the effect of estrogen on TMEFF2 expression was examined. TMEFF2 mRNA levels were up regulated in LNCaP cells by E2 in a dose- and time-dependent manner (**Figure 2**). VD_3 has previously been shown to activate the androgen receptor (AR). VD_3 induced TMEFF2 expression in a dose- and time-dependent manner (**Figure 2**). However, the PPARγ ligands, rosiglitazone and pioglitazone and RXR and RAR ligands, 9-cis retinoic acid and ATRA had no effect on TMEFF2 expression (**Figure 3**). IL-6 and EGF, which have previously been shown to activate AR in an androgen-independent manner, also had no effect on TMEFF2 expression (**Figure 3B**).

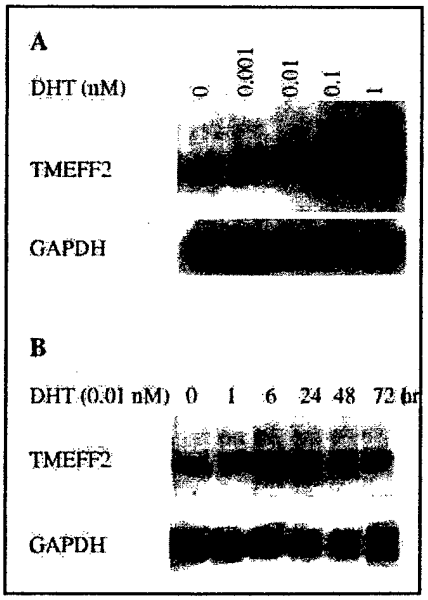

Figure 1. TMEFF2 is regulated by androgen. A dose-dependent effect of dihydrotesterone (DHT) on TMEFF2 expression. LNCaP cells were plated on media supplemented with charcoal stripped PBS for 2 days and then incubated for an additional 2 days with different concentrations of DHT. B Time course of TMEFF2 induction by DHT. LNCaP cells were plated as described in panel A and incubated with 0.01μM DHT for the times indicated.

b. Clone and characterize the TMEFF2 regulatory region.

Complete details of this specific aim can be found in the manuscript entitled, '*Repression of the TMEFF2 promoter by c-myc*' found in the appendix. Here is a brief summary. I cloned the 5'-flanking region of the human TMEFF2 gene and using a luciferase reporter assay showed that it contains a functional promoter. The 0.7-kb region upstream to the TMEFF2 transcription start site

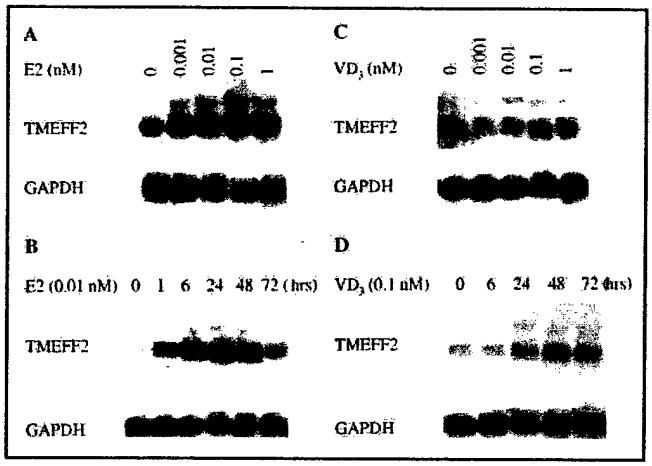

Figure 2. Effect of 17β-estradiol (E2) and 1,25-dihydroxyvitamin D_3 (VD_3) on TMEFF2 expression in LNCaP cells. A. Dose dependent effect of E2 on TMEFF2 expression. B. Time course of TMEFF2 induction by E2. C. Dose dependent effect of VD3 on TMEFF2 expression. D. Time course of TMEFF2 induction by VD3. LNCaP cells were treated with either E2 or VD3 as described in Figure 1.

encompasses the minimal promoter required for TMEFF2 expression. Sequence analysis of the TMEFF2 promoter revealed potential binding sites for several transcription factors including Sp1 and an E-box that could be recognized by c-Myc. An inverse correlation between TMEFF2 and c-Myc expression was found in CWR22 prostate xenografts. Reporter gene and mobility shift assays demonstrated that c-Myc could repress TMEFF2 gene expression through its cognate site. In light of the probable role of TMEFF2 in inhibiting cell growth, its suppression may contribute to the oncogenic properties of c-Myc.

c. Examine the effect of over-expression of HER-2/neu on TMEFF2 expression in LNCaP cells

Although preliminary experiments indicated that TMEFF2 expression was upregulated in LNCaP/Her-2 cells compared to vector control cells, in additional experiments expression levels were not found to be upregulated (**Figure 4**). Therefore no further studies were performed.

d. Explore possible use of TMEFF2 as a biomarker

I have expressed two GST-TMEFF2 fusion proteins in bacteria, purified them on a glutathione column and am in the process of generating antibodies against TMEFF2 in rabbits.

Task 2: Explore the role of TMEFF2 in the proliferation of prostate cancer cells

a. Identify proteins that interact with TMEFF2

I am currently using Matchmaker LexA 2-hybrid system (Clontech) to identify proteins that bind TMEFF2. I have isolated 3 separate domains of TMEFF2: EGF and the two follistatin motifs. I have cloned these into an expression vector and am in the process of screening both a human prostate and human brain Matchmaker cDNA library.

b. Test the effect of overexpression of TMEFF2 on proliferation of prostate cancer cells

For these experiments I utilized DU145 sublines that were generated to overexpress TMEFF2, and measured their proliferative rates. I found that the growth rate of four independent DU145/TMEFF2 clones in culture was reduced by 43-66% as compared with DU145, neo control cell lines (transfected with empty vector, **Figure 5A**). I also examined the effect of cell proliferation by clonogenic assays. PC3 cells were transfected with either an empty pcDNA3.1 vector or a pcDNA3.1 vector expressing TMEFF2 and these cells were cultured in the presence of G418. Expression of TMEFF2 resulted in a 34-65% decrease in the number of G418-resistant colonies (**Figure 5B**).

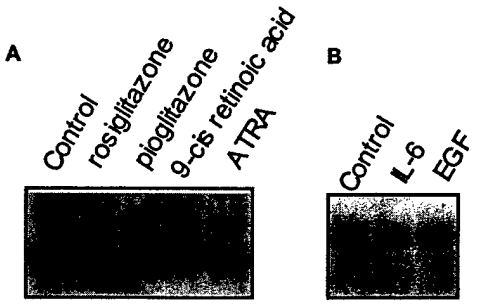

Figure 3 A. Effect of the PPARγ ligands, rosiglitazone, troglitazone and pioglitazone and RXR and RAR ligands, 9-cis retinoic acid and ATRA on TMEFF2 expression in LNCaP cells. B. Effect of the IL-6 and EGF on TMEFF2 expression in LNCaP cells.

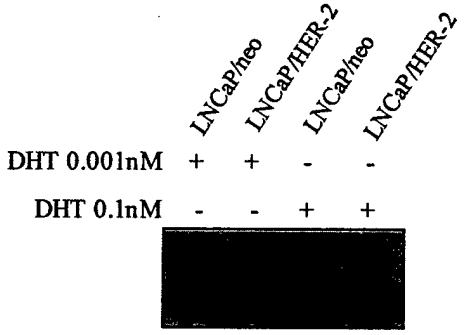

Figure 4 Her-2/neu has no effect on TMEFF2 expression in LNCaP cells. LNCaP/ Her-2 and LNCaP/neo cells were plated in media supplemented with CS PBS for two days, and then incubated with 0.001 or 0.1 nM DHT overnight.

c. Examine the effect of TMEFF2 on the MAPK pathway

I have not yet begun to study this.

d. Correlate and modulate the expression of TMEFF2 in androgen-dependent and androgen-independent prostate cancer xenografts implanted in nude mice and determine if forced expression of TMEFF2 can retard growth of AI prostate cancer cells

I am currently investigating the effects on TMEFF2 expression of castration of male nude mice with CWR22AD xenografts in order to confirm our preliminary data. In addition, I have extracted AI and AD xenografts of LAPC4, LAPC9 and CWR22 grown in male and female mice ± castration ± administration of casedex (male mice) or tamoxifene (female mice) ± administration of estrogen in castrated females ± administration of androgens in castrated males and extracted RNA. I am in the process of preparing Northern blots of this RNA in order to examine expression of TMEFF2, PSA, p53, $p21^{waf1}$ and $p27^{kip1}$.

e. Determine if decreased expression of TMEFF2 during progression to AI in prostate cancer xenografts is correlated with hypermethylation of TMEFF2

I am currently undertaking methylation analysis of several AD and AI human xenografts.

Key Research Accomplishments:

- Expression of TMEFF2 in LNCaP cells is controlled by estrogen and androgen
- Forced expression of TMEFF2 can inhibit growth of prostate cancer cell lines
- TMEFF2 and c-myc expression are inversely correlated in CWR22 prostate xenografts.
- C-myc can repress TMEFF2 expression through its promoter region.

Reportable Outcomes:

Manuscript entitled, *'Repression of the TMEFF2 promoter by c-myc'* submitted, March 2003.

Conclusions: TMEFF2 can inhibit prostate cancer cell growth. Expression of the TMEFF2 gene is controlled by androgen as shown by DHT markedly increasing TMEFF2 expression in LNCaP cells, and by androgen dependent human xenografts, which expressed high levels of TMEFF2 that decreased by day 10 after castration of the mice. C-myc can repress TMEFF2 expression through its promoter region. In light of a probable role of TMEFF2 in inhibiting cell growth, its suppression may contribute to the oncogenic properties of c-Myc.

Appendices: Copy of manuscript, *'Repression of the TMEFF2 promoter by c-myc'.*

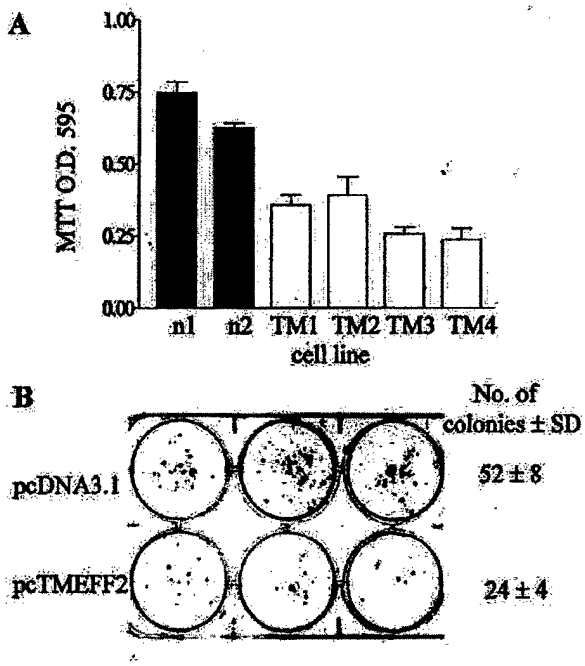

Figure 5 Effect of TMEFF2 on growth of prostate cancer cells. A. MTT assay. Two DU145/neo sublines (n1, n2) and four DU145/TMEFF2 sublines (TMI4) were plated in 96-well plates at a concentration of 1000 cells per well. MTT assays were performed after 4 days. B. Colony formation assay. PC3 cells were transfected with either an empty vector pcDNA3.1 vector or a pcDNA3.1 expressing TMEFF2 and cultured in the presence of G418 (500μg/ml)

Repression of the TMEFF2 promoter by c-Myc

Sigal Gery[1], and H. Phillip Koeffler[1]

Key words: TMEFF2, c-Myc, promoter, transcriptional-suppression and growth inhibition

[1]*Division of Hematology/Oncology, Cedars-Sinai Medical Center, UCLA School of Medicine, Los Angeles, CA 90048*

[1]Corresponding author: Sigal Gery, Division of Hematology/Oncology, Cedars-Sinai Medical Center, Los Angeles, CA 90048, TEL: (310) 423-4609, FAX: (310) 423-0225, E-mail: gerys@schs.org

Abstract

TMEFF2 is a novel transmembrane protein, containing two follistatin domains and an epidermal growth factor-like motif that is mainly expressed in the prostate and brain. Recently, we showed that expression of TMEFF2 could inhibit prostate cancer cell growth. In addition, the TMEFF2 gene is frequently hypermethylated in human tumor cells, suggesting it might be a tumor suppressor gene. We cloned the 5'-flanking region of the human TMEFF2 gene and using a luciferase reporter assay showed that it contains a functional promoter. The 0.7-kb region upstream to the TMEFF2 transcription start site encompasses the minimal promoter required for TMEFF2 expression. Sequence analysis of the TMEFF2 promoter revealed potential binding sites for several transcription factors including Sp1 and an E-box that could be recognized by c-Myc. An inverse correlation between TMEFF2 and c-Myc expression was found in CWR22 prostate xenografts. Reporter gene and mobility shift assays demonstrated that c-Myc could repress TMEFF2 gene expression through its cognate site. In light of the probable role of TMEFF2 in inhibiting cell growth, its suppression may contribute to the oncogenic properties of c-Myc.

Introduction

TMEFF2 (tomoregulin, TPEF, HPP, TENB2) is a recently cloned transmembrane protein containing an altered epidermal growth factor (EGF)-like motif and two follistatin domains, that is predominantly expressed in prostate and brain.[1-5] The function of TMEFF2 is largely unknown, but its structural domains suggest that it may play a role in signaling by growth factors. We have recently shown that forced expression of TMEFF2 can inhibit growth of human prostate cancer cells.[6] In addition, the 5'-region of the TMEFF2 gene is frequently hypermethylated in human tumor cells, suggesting it might be a tumor suppressor gene.[3,5]

The c-Myc protooncogene has been implicated in a variety of cellular processes including proliferation, differentiation and apoptosis, and is involved in many chromosomal abnormalities that play a role in tumorigenesis (reviewed in ref. 7 and 8). Overexpression of c-Myc may contribute to the onset of some cancers including prostate cancer. Myc is a transcription factor, and its role in transformation has been mainly ascribed to the regulation of downstream target genes. c-Myc forms heterodimers with Max that can bind to the E-box sequence CACGTG (as well as related non-canonical sites) and activates transcription of target genes. Among c-Myc inducible genes, ornithine decarboxylase[9] and cdc25A[10] are of interest in this respect because of their involvement in cell cycle progression. Max also forms alternative dimers with the Mad network of proteins that compete with the Myc-Max heterodimers resulting in transcriptional repression. Recent reports have demonstrated that c-Myc repression results not from direct binding to DNA by Myc/Max/Mad dimers, but rather by its interactions with other transcription factors (reviewed in ref. 11 and 12). Interestingly, many of the genes

reported to be downregulated by Myc, like gadd45[13], gas1[14], p21[(WAF1/CIP1),15], p15[INK4b,16] and p27 [kip1,17] are involved in growth arrest.

In this study, we cloned the 5'-flanking region of the human TMEFF2 gene and showed that it contains a functional promoter. We also demonstrated that c-Myc could repress TMEFF2 transcription in prostate cells. Given the relevance of TMEFF2 in slowing cell growth, its suppression may contribute to the oncogenic properties of c-Myc.

Results

Cloning the TMEFF2 promoter region. We have mapped the genomic locus of TMEFF2 to the human chromosome 2q32.1.[6] Searching the Genbank database, we found a human BAC clone from chromosome 2 (PR11-394A2) that was partly homologous to TMEFF2 cDNA. From this BAC, we cloned a 12-kb *Eco*RI fragment that encompassed the 5' genomic region of TMEFF2. To test whether this region has a functional promoter, a 3.5-kb fragment containing upstream TMEFF2 sequences, from –3500 to +31 relative to the TMEFF2 transcription start site,[3] was placed in front of a luciferase reporter gene in the pGL3 basic vector. A significant increase in luciferase activity was observed in the prostate cancer cell lines DU145 (80-fold) and LNCaP (60-fold), transfected with the TMEFF2 promoter-luciferase vector compared to cells transfected with the empty reporter vector (Fig. 1A). Sequence analysis of this region revealed that the TMEFF2 5'-flanking region had potential binding sites for several transcription factors including Sp1 and Myc (E-box), as well as 2 CCAAT-boxes, but no TATA-box was present (Fig. 1B). Similar results were reported in an earlier study where the TMEFF2 promoter had been cloned from a λ genomic library and subjected to sequence analysis. The results from that study also showed that the 5'-region of TMEFF2 contained a CpG island.[3]

Next, to define DNA regions important for gene expression, deletions of the 5'-flanking sequences were constructed and used in reporter gene assays. Deletion to –690 resulted in the highest luciferase activity (85-fold compared to the empty vector, Fig. 1C). Further deletion to –230 and –120 resulted in a lower level of activity. These results suggest that the 0.7-kb region upstream to the TMEFF2 transcription start site contains the minimal promoter of TMEFF2.

TMEFF2 is a possible target gene for c-Myc. A potential c-Myc binding site, 5'-CACGTG-3' (E-box), was identified at position –90 relative to the TMEFF2 transcription start site (+1). Comparison of the murine and human TMEFF2 genes revealed that the murine TMEFF2 genomic upstream region (-75 to the transcription start site) is 92 % homologous to the human TMEFF2 promoter, and that the murine gene also contains an E-box in a similar position (Fig. 2A). We have previously shown that TMEFF2 is expressed in the androgen-dependent CWR22 prostate cancer xenografts and levels are downregulated in the androgen-independent CWR22R xenografts.[6] Northern blot analysis showed that a negative correlation existed between expression of TMEFF2 and c-Myc in the CWR22 xenograft samples (Fig. 2B). These findings suggest that TMEFF2 might be a downstream target for c-Myc.

c-Myc negatively regulates TMEFF2 through the E-box sequence. The effect of c-Myc expression on the TMEFF2 promoter was examined in a reporter assay. DU145 cells, that express endogenous c-Myc, were cotransfected with the 0.7-kb (-690 to +31) TMEFF2 promoter-luciferase construct along with a c-Myc expression plasmid. The results showed that overexpression of c-Myc inhibited luciferase activity by 10-fold compared to cells transfected with an empty expression vector (Fig. 3). Mutations of the E-box (CACGTG → CAGTTG) in the TMEFF2 promoter abolished the inhibitory effect of c-Myc, indicating that the site is essential for negative regulation of TMEFF2 by c-Myc.

The ability of c-Myc to bind specifically to its respective consensus sequence in the TMEFF2 promoter was determined by EMSA using nuclear extracts from LNCaP cells, which express endogenous c-Myc. The results show that a complex from LNCaP cells

could bind the oligonucleotide probe that contained the E-box from the TMEFF2 promoter (Fig. 4, lane 1). The binding was competed by unlabeled homologous oligonucleotides (lanes 2 and 3) but not by mutated oligonucleotides in which the E-box sequences were altered (lanes 4 and 5). The band was also disrupted by addition of antibodies against c-Myc (lane 6). These results indicate that c-Myc can bind to its cognate site in the TMEFF2 promoter.

Discussion

In the present study, we cloned the 5'-flanking region of the human TMEFF2 gene, and analyzed the ability of the TMEFF2 promoter to drive expression of a reporter gene in prostate cancer cells. We demonstrated that the 5'-flanking region contained a functional promoter and c-Myc could repress TMEFF2 promoter activity.

A typical TATA box was not present in the 5'-flanking sequence upstream to the TMEFF2 transcriptional start site. One group of genes that lack the TATA box consists of GC-rich promoters with several Sp1 binding sites.[18] The 5'-region of TMEFF2 is a CpG island and contains several potential Sp1 binding sites.[3,5] TMEFF2 may, therefore, belong to this class of genes.

TMEFF2 is expressed almost exclusively in the prostate and brain.[1-6]. The present study did not identify factors responsible for this high tissue specific expression. Moreover, we have shown that TMEFF2 is an androgen-regulated gene. A sequence with significant homology to the consensus androgen response element (ARE) was not identified within the 3.5-kb region upstream to the TMEFF2 transcriptional start site. An ARE might be present in a further upstream regulatory region. Alternatively, upregulation of TMEFF2 by androgen could be a secondary event. Further work needs to be done to understand the molecular mechanism leading to the tissue specific and androgen-regulated expression of TMEFF2.

Using a reporter assay, we identified a 0.7-kb fragment (–690 to +1) that consists of the TMEFF2 minimal promoter. This region contained several putative transcription factor-binding sites, including the E-box sequence -CACGTG- that could be recognized by Myc. The human and murine TMEFF2 promoters both contain the E-box sequence in

a similar position. In addition, an inverse correlation between TMEFF2 and c-Myc expression was found in CWR22 prostate xenografts. Reporter assays using TMEFF2 minimal promoter and a c-Myc expression vector demonstrated that c-Myc could repress TMEFF2 promoter activity. Moreover, mutation in the E-box abolished c-Myc mediated repression. Furthermore, gel shift experiments showed that c-Myc could bind to the E-box sequence of TMEFF2 and that antibodies against c-Myc could disrupt the binding. These results suggest that c-Myc could inhibit TMEFF2 expression through its cognate site. Results from reporter assays showed that the basal levels of two smaller promoter constructs (-230 to +31 and –120 to +31) were lower than the minimal promoter level (-690 to +31) in the presence of the c-Myc expression vector. This suggests that the region between –690 and –230 may contain sequences that are target for transcriptional repression. Further studies are required to determine the identity of these sequences and the factors that bind them.

Multiple pathways of c-Myc-mediated gene repression have been elucidated[11,12]. In several promoters, gene repression by c-Myc has been mapped to the initiator (Inr) element, indicating that factors that bind at the start site of transcription may be targets for c-Myc activity.[19,20] For example, c-Myc repression of the cyclin dependent kinase inhibitor (CDKI) $p15^{INK4b}$ is mediated through association of Myc to the Miz-1 transcription factor that binds to the Inr element, and this dose not require Max[16]. Downregulation of another CDKI, $p27^{Kip1}$, by c-Myc involves binding of the Myc/Max complex to the Inr element of the $p27^{Kip1}$ promoter[17]. In a third case of Myc-mediated repression of a CDKI, $p21^{(WAF1/CIP1)}$, the mechanism is not mediated via the Inr element or Myc/Max interaction but involves sequestering of the Sp1/Sp3 transcription factors by c-

Myc.[15] Here, we show that c-Myc downregulates TMEFF2 through the E-box site in the TMEFF2 promoter. We did not identify which protein partner binds with c-Myc to facilitate this repression. Since c-Myc cannot bind DNA alone, further studies will need to be done to identify the other protein(s) that interact with c-Myc to inhibit TMEFF2 expression.

Although TMEFF2 function is unknown, its structural domains (two follistatin domains and an EGF-like motiff) suggest that it may have a role in the regulation of growth factor signaling either as a ligand precursor, a membrane-bound receptor or as a binding protein for growth factors. In the EGF-like domain of TMEFF2, a critical arginine residue at position 41 is replaced by a histidine. Replacement of this arginine, results in a drastic decrease of EGF affinity to its receptor.[21] Follistatin domains have been identified in other proteins such as Agrin and Osteonectin where they were shown to bind and neutralize different growth factors including members of the transforming growth factor-β family, platelet-derived growth factor and vascular endothelial growth factor.[22-24] We have previously shown that forced expression of TMEFF2 in prostate cancer cells inhibited their proliferation suggesting that TMEFF2 has a negative effect on growth.[6] Also, two independent studies suggest that TMEFF2 might be a tumor suppressor gene because it is frequently hypermethylated in human tumor cells.[3,5] However, in contrast to these findings, TMEFF2 was shown to promote the survival of specific neurons in primary culture,[2] and a recent report associated TMEFF2 (TENB2) expression with prostate cancer progression.[1] More detailed studies need to be undertaken to understand better the function of TMEFF in normal cells and its potential role in cancer.

c-Myc is a protooncogene that plays a prominent role in various types of cancer including prostate cancer. Overexpression of c-Myc in quiescent cells induces cell cycle entry and deregulated expression of c-Myc induces tumorigenesis. Recent studies suggest that gene repression is essential for c-Myc-induced cell cycle progression and cellular transformation. Repression of cell cycle inhibitory genes, as well as induction of genes with growth stimulating functions, could provide the mechanism by which c-Myc promotes cell growth. We suggest that inhibition of TMEFF2 expression by c-Myc may play a similar role in prostate cells. Further studies will determine the relevance of TMEFF2 suppression to oncogenic transformation.

Materials and Methods

Cloning of the 5'-Flanking Region of the Human TMEFF2 Gene. The human BAC clone PR11-394A2 (GenBank accession number AC092644, Research Genetics) was digested with *Eco*RI and the resulting products were cloned into the pGEM-3Z vector (Promega). The colonies were screened by hybridization to the TMEFF2 probe. A single clone was isolated and a 12-kb insert was characterized by restriction enzyme analysis and partial sequencing. Various size promoter fragments were amplified by PCR and cloned into the promoterless luciferase reporter vector, pGL3 basic (Promega). Mutations in the c-Myc binding site were introduced using the GeneEditor In Vitro site Directed Mutagenesis System (Promega).

Prostate Xenografts and Cell Culture. The prostate cancer xenograft CWR22 has been described.[25] The prostate cancer cell lines LNCaP, and DU145 were obtained from the American Type Culture Collection and grown in the recommended medium and conditions.

Transfections and Luciferase Assay. Cells were transfected using the GenePORTER Transfection Reagent (GTS Inc.) with 1 μg of each promoter-luciferase construct. In co-transfection experiments, 1 μg of c-Myc expression vector[26] (pMV6/c-Myc which contains the human c-Myc inserted between *Eco*RI and *Hind*III sites in the pMV6 vector) was transfected with the promoter-luciferase construct. Lysates were harvested 48 hours post-transfection and luciferase activity was measured with the Dual-Luciferase reporter 1000 assay system (Promega). Transfection efficiency was normalized using 0.1 μg pRL-SV40.

Northern blot analysis. Total RNA was isolated from prostate cancer xenografts using TRIzol (Life Technologies, Inc.). For Northern analysis, 10 μg of total RNA was fractionated on 1.2 % agarose denaturing gels and transferred to nylon membranes (Amersham). Probes were labeled with the Strip-EZ DNA kit (Ambion) and hybridizations were performed in the ULTRAhyb buffer (Ambion) according to the manufacture's instructions.

Electrophoretic mobility shift assay (EMSA). Double stranded oligonucleotides were end-labeled with γ-^{32}P-ATP by T4 polynucleotide kinase. The oligonucleotide sequences were as follow: wild type oligonucleotide; 5'-TGCCTCTTCC**CACGTG**ACCCCGGG CG-3', mutant oligonucleotide (used in competition experiments); 5'-TGCCTC TTCC**CAGTTG**ACCCCGGGCG-3'. Nuclear extracts were prepared from LNCaP cells with the CelLytic Nuclear extraction Kit (Sigma). Ten μg of nuclear extract proteins were incubated with 20,000 cpm of labeled wild type probe. Binding reactions were incubated for 30 min on ice and then analyzed on 4 % polyacrylamide gel. When cold competitor or a c-Myc antibody (sc-764X, Santa Cruz Biotechnology) were used, they were added to the reactions 20 min prior to the labeled probe.

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Figures

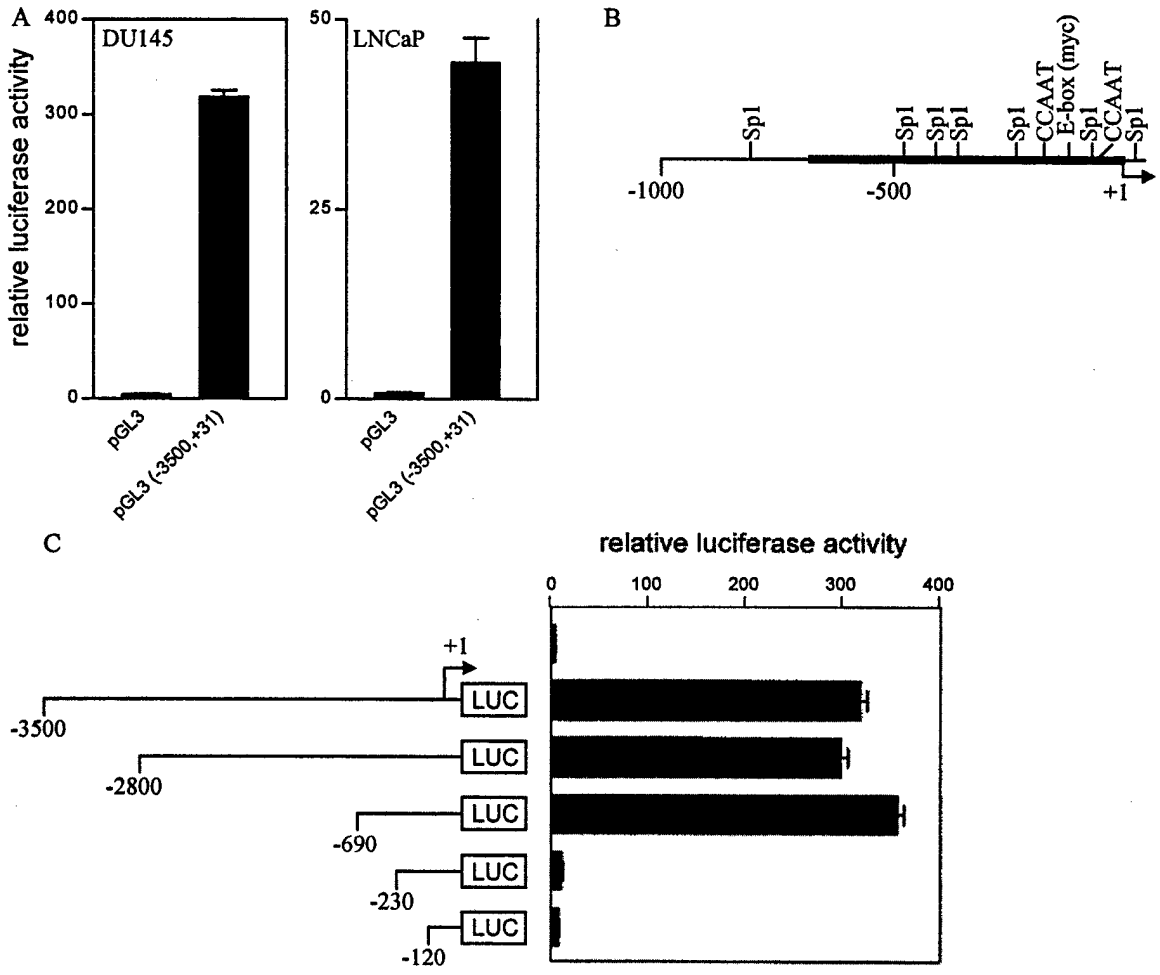
Fig. 1. Promoter activity of the 5'-flanking region of the human TMEFF2 gene. *A*, The 5'-flanking 3.5-kb (-3500 to +31) DNA fragment of TMEFF2 was subcloned into the luciferase reporter gene in the pGL3 vector. DU145 and LNCaP prostate cancer cells were cotransfected with either 1 μg of the pGL3 (-3500 to +31) promoter-luciferase vector or the pGL3 empty vector, along with 0.1 μg of the pRL-SV40 vector that served as internal control for transfection efficiency. *B*, Schematic presentation of the 5' region of the TMEFF2 gene. The thick line represents the minimal promoter. *C*, Deletions of the 5'-flanking 3.5-kb fragment of TMEFF2 were made and subcloned into the luciferase reporter gene in the pGL3 vector. DU145 cells were cotransfected with 1 μg of either one of the various pGL3 promoter-luciferase reporter constructs or an empty pGL3 vector, together with 0.1 μg of the pRL-SV40 vector. After two days, cells were harvested and luciferase activity was measured. Results represent the mean ± SD of triplicate transfections. The experiments were repeated three times with triplicate plates per experiment point.

Fig. 2. TMEFF2 is a potential target gene for c-Myc. *A,* Comparison between the human and murine potential c-Myc binding site (E-box) in the TMEFF2 promoter. The E-box sequence is underlined and is in bold letters; the upper arrow represents the human (GenBank accession number AC092644) transcription starting point; the lower arrow displays the murine (GenBank accession number AC098743) transcription-starting site. *B,* TMEFF2 expression inversely correlates with c-Myc expression in CWR22 xenografts. RNA from androgen-dependent (CWR22) and androgen-independent

(CWR22R) xenografts was electrophoresed, and Northern blot was performed using radiolabeled TMEFF2 cDNA probe. The blots were rehybridized with a control GAPDH probe. The blot was stripped and reprobed with α^{32}P-dATP-labeled cDNA probes for c-Myc and GAPDH.

Fig. 3. c-Myc represses expression of the TMEFF2 promoter. DU145 cells were cotransfected with the 0.7-kb (-690 to +31) fragment of the TMEFF2 promoter-luciferase and either a pMV6/c-Myc expression vector or an empty pMV6 vector and 0.1 μg of the pRL-SV40 vector. Cells were harvested and luciferase activity was measured as described in Fig. 1. Results represent the mean ± SD of triplicate transfections. The experiments were repeated three times with triplicate plates per experimental point.

Fig. 4. c-Myc binds to the c-Myc binding site in the TMEFF2 promoter. EMSA was performed using 10 μg of nuclear extract proteins from LNCaP cells. Extracts were incubated with ^{32}P-labeled wild type oligonucleotide containing the E-box site (CACGTG) from the TMEFF2 promoter (-100 to –78). Unlabeled wild type competitor (wt competitor), mutant c-Myc competitor (containing a mutation in the E-box; CACGTG → CAGTTG, mut competitor) or a c-Myc antibody were added to the reactions as indicated.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DU145
LNCaP
relative luciferase activity
400
300
200
100
0
50
25
0
pGL3
pGL3 (-3500,+31)
B
Sp1
CCAAT
E-box (myc)
-1000
-500
+1
C
relative luciferase activity
0
100
200
300
400
+1
LUC
-3500
-2800
-690
-230
-120

A

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Human  -110  agagcccgactgcctcttccacgtgaccccgggcgggctgaggacctgctgcttcccaa  -51
             ||||  |||||||| ||||||||||||||||||||||| ||||||||||||||||||
Murine  -75  agagtccgactgccgcttccacgtgaccccgggcgggctaaggacctgctgcttcccaa  -16

Human   -50  acgccagagggatgcgggcggcagagctcgagaggcggctgcccgggctgcgggggcgctgt  +10
             |||||||||||||||||||||||||| ||||||||||||| ||||||||||||| |
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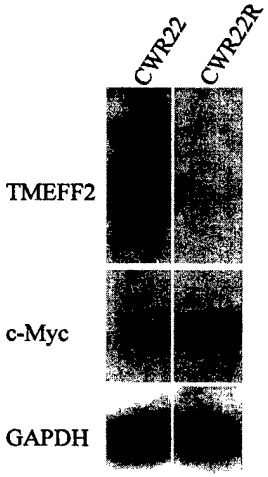
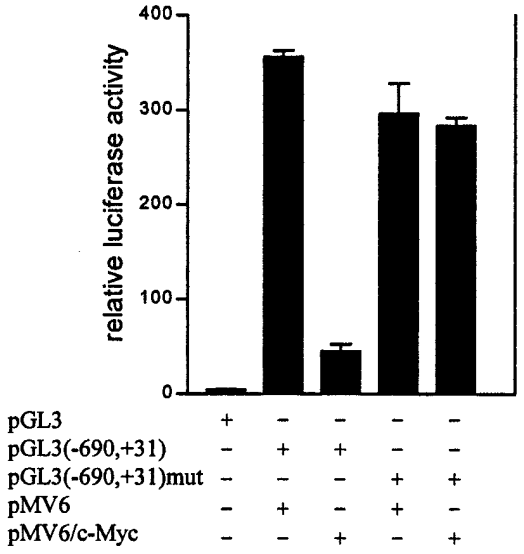
B




relative luciferase activity
400
300
200
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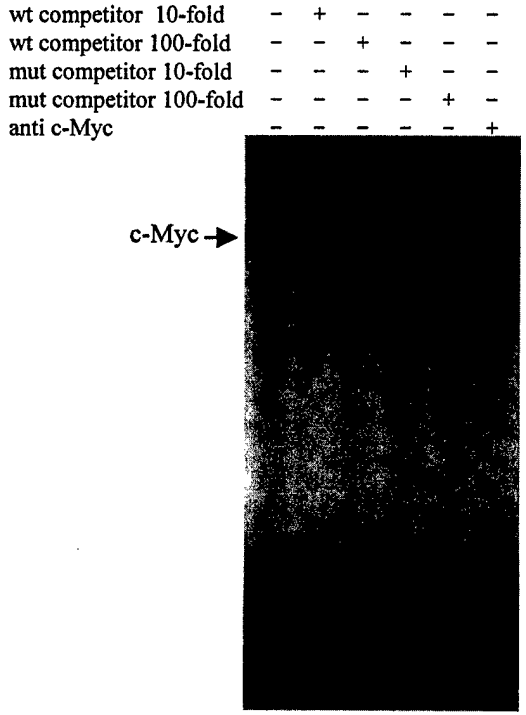
pGL3 + − − − −
pGL3(-690,+31) − + + − −
pGL3(-690,+31)mut − − − + +
pMV6 − + − + −
pMV6/c-Myc − − + − +


wt competitor 10-fold - + - - - -
wt competitor 100-fold - - + - - -
mut competitor 10-fold - - - + - -
mut competitor 100-fold - - - - + -
anti c-Myc - - - - - +
c-Myc